J Nat Med (2010) 64:117–120
DOI 10.1007/s11418-009-0372-2

NATURAL RESOURCE LETTER

Antiviral activities of diarylheptanoids against influenza virus in vitro

Rie Sawamura · Yi Sun · Ken Yasukawa · Tomomi Shimizu · Wataru Watanabe · Masahiko Kurokawa

Received: 17 August 2009 / Accepted: 8 October 2009 / Published online: 7 November 2009

Abstract The anti-influenza A/PR/8/34 (H1N1) virus activities of ten diarylheptanoids isolated from *Alpinia officinarum* were examined using the MTT method. The 50% inhibitory concentration of each diarylheptanoid examined was clearly lower than its 50% cytotoxic concentration determined by the MTT assay and/or maximum non-cytotoxic concentration (MNCC) determined by the morphological change of cells. In particular, the influenza virus was more susceptible to 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-4*E*-hepten-3-one (**3**) and (5*S*)-5-hydroxy-7-(4″-hydroxyphenyl)-1-phenyl-3-heptanone (**8**) than the other diarylheptanoids. Thus, all diarylheptanoids exhibited potential antiviral activity against influenza virus in vitro.

Keywords *Alpinia officinarum* · Diarylheptanoid · Antiviral activity · Influenza virus

Introduction

The pandemic influenza H1N1 virus has recently spread worldwide. The appearance of an influenza virus more virulent than the pandemic H1N1 is now predicted. The amantadine and neuraminidase inhibitors zanamivir and oseltamivir have been used for the treatment and prevention of influenza virus infection [1–3], but the appearance of viruses resistant to them has been reported [1, 4–6]. It is therefore important to develop new types of anti-influenza virus agents with anti-influenza virus actions different from those of the known agents.

In a series of studies on the development of bioactive components from natural sources, we found that a methanol extract from the rhizome of *Alpinia officinarum* is effective in inhibiting tumor promotion in 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammatory ear edema in mice [7]. Diarylheptanoids isolated from *Alpinia officinarum* have been shown to exhibit biological activities such as antioxidant activity [8, 9], cytotoxic activity [10], suppressive activity of inducible nitric oxide synthase expression [11], and inhibitory activity of biosynthesis of prostaglandin and leukotriene [12, 13]. Although a variety of biological activities associated with diarylheptanoids have been demonstrated, antiviral activity by diarylheptanoids has not been reported. In the present study, we examined the potential anti-influenza virus activity of diarylheptanoids in vitro.

Materials and methods

Chemicals

Bovine serum albumin was purchased from Sigma–Aldrich Japan, Tokyo, Japan. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma, Japan. Sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide, and ribavirin

R. Sawamura · T. Shimizu · M. Kurokawa (✉)
Department of Biochemistry, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, 1714-1 Yoshino, Nobeoka, Miyazaki 882-8508, Japan
e-mail: b2mk@phoenix.ac.jp

Y. Sun · K. Yasukawa
College of Pharmacy, Nihon University, 7-7-1, Narashinodai, Funabashi, Chiba 274-8555, Japan

W. Watanabe
Department of Microbiology, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, 1714-1 Yoshino, Nobeoka, Miyazaki 882-8508, Japan

were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Diarylheptanoids (**1**–**10**) were isolated from the rhizome of *Alpinia officinarum* as described previously [10, 14].

Cell and viruses

Madin–Darby canine kidney (MDCK) cells, which were provided by Dr. H. Ochiai (Toyama University, Japan), were grown and maintained in Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 8 and 2% heat-inactivated fetal calf serum, respectively [15]. The influenza virus A/PR/8/34 (H1N1) was provided by Dr. H. Ochiai (Toyama University, Japan) and propagated in MDCK cells [15].

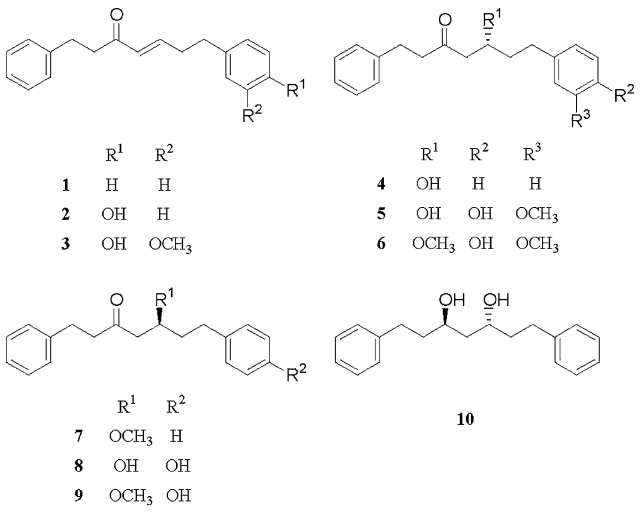

Fig. 1 Structures of diarylheptanoids (**1**–**10**) from the rhizomes of *Alpinia officinarum*

Antiviral and cytotoxic assay

The anti-influenza virus activity of 10 diarylheptanoids was examined using the MTT assay. Briefly, MDCK cells grown in 96-well plates were infected with 60 plaque-forming units/50 μl of influenza A/PR/8/34 (H1N1) virus at 37°C for 1 h. The cells were overlaid with 50 μl of maintenance EMEM containing 0.1% bovine serum albumin and various concentrations (0, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 50 μg/ml) of one of the following compounds, and maintained in a humidified atmosphere containing 5% CO_2 for 3–4 days. All diarylheptanoids were dissolved in DMSO and diluted with culture medium to make the various final concentrations. The concentration of DMSO in each medium was less than 1%. Ribavirin was dissolved in distilled water and used as a control. For the MTT assay, 10 μl of MTT (7.5 mg/ml) solution in phosphate-buffered saline was added to octuplicate wells with each concentration of diarylheptanoids and incubated for 4 h at 37°C in a CO_2 incubator. The crystallized formazan in the plates was dissolved by the addition of 100 μl of 20% (w/v) SDS/50% (v/v) *N*,*N*-dimethylformamide. Absorbance was measured at two wavelengths (540 and 690 nm) in a

Table 1 Anti-influenza virus activity and cytotoxicity of diarylheptanoids

Compounds	EC_{50}^{a} (μg/ml)	Cytotoxicity (μg/ml)		CC_{50} or MNCC/EC_{50}
		CC_{50}^{b}	MNCC[c]	
1,7-Diphenyl-4*E*-hepten-3-one (**1**)	<30	>80	>50	>2.7
7-(4″-Hydroxyphenyl)-1-phenyl-4*E*-hepten-3-one (**2**)	<10	ND	>30	>3.0[d]
7-(4″-Hydroxy-3″-methoxyphenyl)-1-phenyl-4*E*-hepten-3-one (**3**)	2.9 ± 0.3	34.0 ± 0.6	>30	>11.7
(5*R*)-5-Hydroxy-1,7-diphenyl-3-heptanone (**4**)	11.7 ± 0.8	52.8 ± 0.1	>30	>4.5
(5*R*)-5-Hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**5**)	22.4 ± 1.8	>80	>50	>3.6
(5*R*)-5-Methoxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**6**)	<10	36.4 ± 0.5	>30	>3.6
(5*S*)-5-Methoxy-1,7-diphenyl-3-heptanone (**7**)	6.1 ± 0.5	>80	>50	>13.1
(5*S*)-5-Hydroxy-7-(4″-hydroxyphenyl)-1-phenyl-3-heptanone (**8**)	0.7 ± 0.3	>80	>50	>114.3
(5*S*)-5-Methoxy-7-(4″-hydroxyphenyl)-1-phenyl-3-heptanone (**9**)	7.0 ± 0.2	ND	>30	>4.3[d]
(3*R*,5*R*)-1,7-Diphenyheptan-3,5-diol (**10**)	15.1 ± 0.9	ND	>50	>3.3[d]
Ribavirin	16.7 ± 0.4	>50	ND	>3.0

ND Not done

[a] Mean ± SE for an experiment with octuplicate wells

[b] Mean ± SE for three independent experiments were determined by MTT assay

[c] The highest concentration of diarylheptanoids at which the obvious morphological change was not detected in more than 50% of octuplicate wells under microscopic observation

[d] MNCC values were used

computer-controlled microplate reader (Bio-Rad, Tokyo, Japan). The 50% effective antiviral concentration (EC_{50}) was the concentration that reduced virus-induced cell destruction by 50%, as described previously [16]. The cytotoxicity of diarylheptanoids (**1** and **3–8**) and ribavirin was assessed by MTT assay using mock-infected MDCK cells exposed to each diarylheptanoid (0, 15, 30, 50 or 45, 60, and 80 μg/ml) in duplicate wells for 3–4 days as described above. The 50% cytotoxic concentration (CC_{50}) was determined as the concentration that reduced cell destruction by 50% in the three independent experiments [16]. The cytotoxicity of all diarylheptanoids used was also assessed by the detection of morphological changes in the infected cells under the microscope. The maximum non-cytotoxic concentration (MNCC) of diarylheptanoids was defined as the highest concentration at which obvious morphological change was not observed in more than 50% of octuplicate wells.

Results and discussion

Diarylheptanoids (**1–10**, Fig. 1) were examined for their anti-influenza virus activity and cytotoxicity in vitro. As shown in Table 1, the EC_{50} value of each diarylheptanoid examined for influenza virus was clearly lower than the CC_{50} and/or MNCC of each diarylheptanoid. DMSO at 1%, which was used as a maximum concentration to dissolve diarylheptanoids in the culture medium, was not cytotoxic. The therapeutic indexes (CC_{50}/EC_{50}) of 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-4*E*-hepten-3-one (**3**) and (5*S*)-5-hydroxy-7-(4″-hydroxyphenyl)-1-phenyl-3-heptanone (**8**) were more than 11.7 and 114.3, respectively, and influenza virus was more susceptible to **3** and **8** than to the other diarylheptanoids. In this assay, the EC_{50} value of ribavirin, used as a control, was similar to the results reported previously [15, 17]. The EC_{50} values of the diarylheptanoids examined were similar to or lower than that of ribavirin, and they were demonstrated to show potential antiviral activity against influenza virus in vitro. This is the first evidence demonstrating the anti-influenza virus activity of diarylheptanoids in vitro. Various kinds of diarylheptanoids have been isolated from *Alpinia officinarum* [10, 12–14, 18, 19]. Studies of the relationship between their structures and their anti-influenza activities may be worthwhile to obtain much more effective anti-influenza virus diarylheptanoids.

Acknowledgments We thank Ms. Akane Hino for her excellent technical assistance and Ms. Katherine Ono for her editorial assistance. This study was partly supported by Grants-in-Aid for Scientific Research (No. 20590131) and for Young Scientists (No. 20790430) from the Japan Society for the Promotion of Science and Health and Labor Sciences Research Grants (Research on Risk of Chemical Substances) from the Ministry of Health, Labor and Welfare of Japan.

References

1. Hayden FG (1996) Amantadine and rimantadine—clinical aspects. In: Richman DD (ed) Antiviral drug resistance. Wiley, London, pp 59–77
2. Ison MG, Hayden FG (2001) Therapeutic options for the management of influenza. Curr Opin Pharmacol 1:482–490
3. Pitts SR (2002) Use of the neuraminidase inhibitor class of antiviral drugs for treatment of healthy adults with an acute influenza-like illness. Ann Emerg Med 39:552–554
4. Englund JA, Champlin RE, Wyde PR, Kantarjian H, Atmar RL, Tarrand J, Yousuf H, Regnery H, Klimov AI, Cox NJ, Whimbey E (1998) Common emergence of amantadine- and rimantadine-resistant influenza A viruses in symptomatic immunocompromised adults. Clin Infect Dis 26:1418–1424
5. McKimm-Breschkin J, Trivedi T, Hampson A, Hay A, Klimov A, Tashiro M, Hayden F, Zambon M (2003) Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. Antimicrob Agents Chemother 47:2264–2272
6. Wetherall NT, Trivedi T, Zeller J, Hodges-Savola C, McKimm-Breschkin JL, Zambon M, Hayden FG (2003) Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. J Clin Microbiol 41:742–750
7. Yasukawa K, Sun Y, Kitanaka S, Tomizawa N, Miura M, Motohashi S (2008) Inhibitory effect of the rhizomes of *Alpinia officinarum* on TPA-induced inflammation and tumor promotion in two-stage carcinogenesis in mouse skin. Nat Med 62:374–378
8. Ly TN, Shimoyamada M, Kato K, Yamauchi R (2003) Isolation and characterization of some antioxidative compounds from the rhizomes of smaller galanga (*Alpinia officinarum* Hance). J Agric Food Chem 51:4924–4929
9. Ly TN, Shimoyamada M, Kato K, Yamauchi R (2004) Antioxidative compounds isolated from the rhizomes of smaller galanga (*Alpinia officinarum* Hance). Biofactors 21:305–308
10. Sun Y, Tabata K, Matsubara H, Kitanaka S, Suzuki T, Yasukawa K (2008) New cytotoxic diarylheptanoids from the rhizomes of *Alpinia officinarum*. Planta Med 74:427–431
11. Lee HJ, Kim JS, Ryu J-H (2006) Suppression of inducible nitric oxide synthase expression by diarylheptanoids from *Alpinia officinarum*. Planta Med 72:68–71
12. Kiuchi F, Shibuya M, Sankawa U (1982) Inhibitors of prostaglandin biosynthesis from *Alpinia officinarum*. Chem Pharm Bull 30:2279–2282
13. Kiuchi F, Iwakami S, Shibuya M, Hanaoka F, Sankawa U (1992) Inhibition of prostaglandin and leukotriene biosynthesis by gingerols and diarylheptanoids. Chem Pharm Bull 40:387–391
14. Sun Y, Matsubara H, Kitanaka S, Yasukawa K (2008) Diarylheptanoids from the rhizomes of *Alpinia officinarum*. Helv Chim Acta 91:118–123
15. Shimizu T, Hino A, Tsutsumi A, Park YK, Watanabe W, Kurokawa M (2008) Anti-influenza virus activity of propolis in vitro and its efficacy against influenza infection in mice. Antivir Chem Chemother 19:7–13

16. Watanabe W, Konno K, Ijichi K, Inoue H, Yokota T, Shigeta S (1994) MTT colorimetric assay system for the screening of anti-orthomyxo- and anti-paramyxoviral agents. J Virol Methods 48:257–265
17. Furuta Y, Takahashi K, Fukuda Y, Kuno M, Kamiyama T, Kozaki K, Nomura N, Egawa H, Minami S, Watanabe Y, Narita H, Shiraki K (2002) In vitro and in vivo activities of anti-influenza virus compound T-705. Antimicrob Agents Chemother 46:977–981
18. Shin D, Kinoshita K, Koyama K, Takahashi K (2002) Antiemetic principles of *Alpinia officinarum*. J Nat Prod 65:1315–1318
19. An N, Xu L-Z, Zou Z-M, Yang S-L (2006) Diarylheptanoids from *Alpinia officinarum*. J Asian Nat Prod Res 8:637–641